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MMP-9 protein level does not reflect overall MMP activity in the airways of patients with COPD

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Summary

Background: Animal and human studies have implicated an imbalance of matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) in the pathogenesis of chronic obstructive pulmonary disease (COPD). MMP-9 protein is increased in COPD and we hypothesized that total MMP activity would be raised although this has not previously been measured.

Methods: Using fluorescence and biotin labelled MMP assays, RT-PCR, western blotting and enzyme-linked immunosorbent assay we examined total MMP activity, specific gelatinase, elastase, collagenase activity, TIMP-1 and TIMP-2 in induced sputum from smokers with COPD and smokers without COPD.

Results: Induced sputum was obtained from 15 smokers with COPD and 14 smokers without COPD. MMP-9 levels were higher in those with COPD compared with controls ($p < 0.05$). Total MMP activity, specific gelatinase, collagenase and elastase activities were not higher in COPD patients. In addition, reduced MMP activity was correlated with increasing airflow obstruction in COPD ($p = 0.016$).

Conclusion: MMP-9 protein but not MMP activity was higher in sputum of COPD patients compared with controls. These results suggest that MMP-9 levels may not reflect the overall MMP activity in the airways of patients with COPD suggesting a complex relationship between MMP-9 protein and activity. Further studies of MMPs in COPD should comprise activity measures in addition to protein levels.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality with 75% of cases attributable to smoking.¹ The protease–antiprotease hypothesis has dominated thinking about COPD since the observation that individuals with a hereditary deficiency of the protein α -1 antitrypsin were at increased risk of developing emphysema.² Evidence from animal^{3–8} and human studies^{9–20} has implicated an imbalance of matrix metalloproteinases (MMPs) and their natural inhibitors, tissue inhibitors of matrix metalloproteinases (TIMPs), in COPD pathogenesis.

MMPs are a family of structurally related metalloendopeptidases, which are crucial for the homeostasis and turnover of the extracellular matrix both in health and disease. MMP activity is tightly regulated at the level of gene transcription, by proteolytic activation of the inactive proenzymes and by inhibition of the active enzyme by TIMPs.

Studies have demonstrated increased levels of several MMPs in lung tissue,^{9,12,15} bronchoalveolar lavage (BAL)^{11,12,17,19,20} and induced sputum^{10,13,14,16,18} from patients with COPD. Many have shown that MMP-9 is increased in the airways and lung parenchyma of patients with COPD.^{11–20} These studies have estimated MMP activity either from single MMP/TIMP ratios,^{11,16} by specific immunocapture assays¹³ or zymography.^{11,12,14,17,19,20} We hypothesized that the increase in MMP-9 protein would lead to an increase in overall MMP activity in induced sputum from subjects with COPD, and in contrast to previous studies, examined total MMP activity using a quenched fluorescence assay which is dependent on the total MMP/inhibitor balance.

Methods

Study subjects

The Nottingham Research Ethics Committee approved the study. We recruited smokers with COPD defined by GOLD criteria (COPD) and smokers without COPD (HS). Patients were aged 40–80 years, had at least a 10-pack year smoking history and had not required antibiotics or oral steroids for the previous 6 weeks. Patients with α 1-antitrypsin deficiency, radiological evidence of interstitial lung disease, previous thoracic surgery, or taking inhaled corticosteroids were excluded. Spirometry was performed pre and post bronchodilator according to American Thoracic Society guidelines and breathlessness was recorded using the Medical Research Council (MRC) dyspnoea score. Those with reversibility greater than 10% of baseline FEV₁ after inhaling 400 mcg of salbutamol were excluded. Subjects with FEV₁/FVC of less than 70% were classified as having COPD. Induced sputum samples were obtained from 15 current smokers with COPD (COPD) and 14 current smokers without COPD (HS).

Sputum induction and processing

Induced sputum was collected using previously described techniques.^{21,22} The samples were divided into two portions: one processed with 0.1% dithiothreitol (DL-Dithiothrei-

tol, Sigma),²² the second sonicated. Differential cell counts were performed according to standard methods.

Quenched fluorescence assay for total MMP activity

Total MMP activity was assessed directly by cleavage of the quenched fluorescent substrate (Mca-Pro-Leu-Gly-Dpa-Ala-Arg-NH₂) (R&D Systems, UK) allowing simultaneous measurement of the activity of MMP-1, MMP-2, MMP-7, MMP-8, MMP-9, MMP-12 and MMP-13.²⁴ DTT processed sputum was used. To validate the assay for these samples a linear relationship between fluorescence and concentration of activated recombinant MMP-9 was demonstrated in the presence of DTT.

To ensure the specificity of the assay for MMPs, samples from the HS and COPD groups were incubated with different combinations of selective protease inhibitors. These were, Ilomastat (Calbiochem, Merck Biosciences, Germany) a specific MMP inhibitor, Complete Mini EDTA free Protease Inhibitor Cocktail tablets (Roche Applied Science, Germany) that inhibit serine, cysteine and acidic proteases but not MMPs, and EDTA which inhibits proteins dependent upon divalent cations including MMPs. Due to the volumes of sample required these experiments were performed in BAL fluid obtained from a different study.

Specific activity assays and immunoassay

Collagenase, gelatinase (Chemicon International, CA, USA) and elastase (Molecular Probes, OR, USA) activities were assessed in DTT processed sputum using specific assays. The collagenase assay used bovine biotinylated collagen, which was cleaved by active MMP-1, MMP-8 and MMP-13. The gelatinase assay utilized a biotinylated gelatinase, which was cleaved by active MMP-2 and MMP-9. The elastase assay contains soluble bovine neck ligament elastin that has been labelled with quenched fluorescence molecule. TIMP-1 protein was measured by ELISA (Amersham Bioscience, NJ, USA).

RtPCR

TIMP-1 and TIMP-2 mRNA were detected in sputum cell pellets by RtPCR. RNA was extracted from the stored cell pellets using Qiagen RNA extraction kit (Qiagen, UK), quantified and the purity checked using NanoDrop[®] ND-1000 UV-Vis spectrophotometer. cDNA synthesis was undertaken using Protoscript cDNA synthesis kit (New England Biolabs, MA, USA). PCR was performed using the following primers: TIMP-1 forward AAGGCTCTGAAAGGGCTTC, reverse GAAAGATGGGCGTGGGAACA; TIMP-2 forward CCAAGCAGGAGTTTCTCGAC, reverse GACCCATGGGATGAGTGTTT. The products were run on 2% agarose gel and the bands were sequenced to confirm the identity of the PCR products.

Western blot analysis

Expression of TIMP-1 and TIMP-2 protein was determined by Western blot analysis. Membranes were blocked in 5% dry milk plus unconjugated anti-human IgG (Vector Labs,

Peterborough, UK) and incubated overnight at 4°C with monoclonal TIMP-1 (1:200 dilution) and monoclonal TIMP-2 (1:20 dilution) antibodies (Oncogene, Merck Biosciences, Germany). Recombinant TIMP-1 and TIMP-2 were used as controls (Oncogene, Merck Biosciences, Germany).

Zymography

Supernatants from induced sputum processed by sonication were electrophoresed using pre-cast zymogram gels (Invitrogen, Carlsbad, CA, USA) and processed according to the manufacturers guidelines. Bands were quantified by Gene-tools Software (Syngene, UK).

Statistical analysis

Comparison by group was made using one-way ANOVAs (if normally distributed) or paired *t*-test. A log transformation was used to normalize the positively skewed distribution of total MMP activity, and thus allow adjustment for age and pack years in linear regression models. Correlations were evaluated using the Pearson product-moment correlation. All analyses were performed in SPSS (version 12.0.1 for windows, SPSS Inc., IL, USA). $p < 0.05$ was considered statistically significant.

Results

Subject characteristics

Patients with COPD had mild to severe airflow obstruction, whereas HS had, by definition, normal FEV₁ and FEV₁/FVC ratio > 70. Those with COPD were older and had smoked more than the HS group (Table 1). No significant differences in sputum differential cell counts were found between the two groups (Table 2).

MMP-9 levels in induced sputum and BAL

We examined MMP-9 protein in induced sputum using zymography to confirm findings previously published.^{10,13,14} The MMP-9 band volume was significantly greater in the COPD patients as compared to the HS (Figure 1).

Table 2 Concentrations and differentials of cells in induced sputum in subjects with chronic obstructive pulmonary disease (COPD) and smokers without COPD (HS).

	COPD	HS
Subjects, (n)	15	14
Weight of selected sputum (g)	0.7 (0.65)	0.46 (0.37)
TCC (10 ⁶ cells g ⁻¹)	2.25 (2.46)	2.8 (2.77)
Squamous cells (%)	3.9 (4.55)	2 (3.18)
Nonsq. epith. (%)*	0.7 (0.6)	0.75 (0.68)
Macrophages (%)*	24 (18)	28.5 (10.5)
Neutrophils (%)*	73 (25)	69 (12.75)
Lymphocytes (%)*	0.6 (0.75)	0.5 (0.36)
Eosinophils (%)*	0.5 (0.64)	0.5 (1.45)

Data presented as median (IQR). TCC: total cell count; nonsq. epith.: nonsquamous epithelial cell.

*Expressed as total of non-squamous cells.

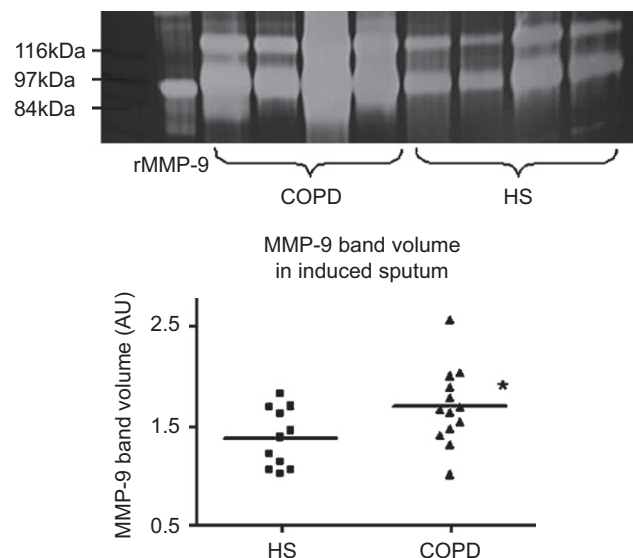


Figure 1 Gelatin zymography of sonicated sputum. Zones of negative staining indicating enzymic activity. rMMP-9 = recombinant activated MMP-9. MMP-9 band volume, derived from zymography, in induced sputum obtained from smokers with COPD (COPD) and smokers without COPD (HS). Solid line = mean value; * $p < 0.05$ vs. HS.

Table 1 Characteristics of study group.

	Age	Gender		FEV ₁ (%predicted)	FEV ₁ /FVC	Pack years	MRC score	Smoking status	
		M	F					Current	Ex-smoker
<i>Median (IQR)</i>									
COPD (<i>n</i> = 15)	62* (16.5)	12	3	64* (43)	58* (20)	50* (28)	2.5 (2.25)	15	0
HS (<i>n</i> = 14)	49 (15)	9	5	90.5 (22.75)	74 (6.5)	38 (16.5)	2 (1)	14	0

* $p < 0.05$ for comparison of COPD group (COPD) with smokers without COPD (HS).

Validation of quench fluorescent assay in sputum and BAL

To test the specificity of the assay for MMP activity we examined the effect of MMP inhibitors on the activity of recombinant MMP proteins and patient samples. These experiments were performed in BAL from another study due to the quantity of sample required. Ilomastat and EDTA strongly inhibited the activity of recombinant MMP-9. EDTA fully inhibited proteolytic activity in BAL although ilomastat

did not (Figure 2a). Complete mini EDTA-free protease inhibitor tablets did not reduce activity in either case suggesting the assay was detecting only metalloproteinase activity (Figure 2a). Further experiments showed that ilomastat inhibited activity in a dose-dependent manner in BAL from healthy smokers and from COPD (Figure 2b). To investigate why ilomastat did not fully inhibit BAL proteolytic activity we examined the effect of ilomastat on recombinant MMP-2, MMP-9 and MMP-3 in the assay. As would be expected from the inhibition constants (K_i) of the inhibitor for these substrates, ilomastat strongly inhibited MMP-2 and MMP-9 and less strongly MMP-3 (Figure 2c).

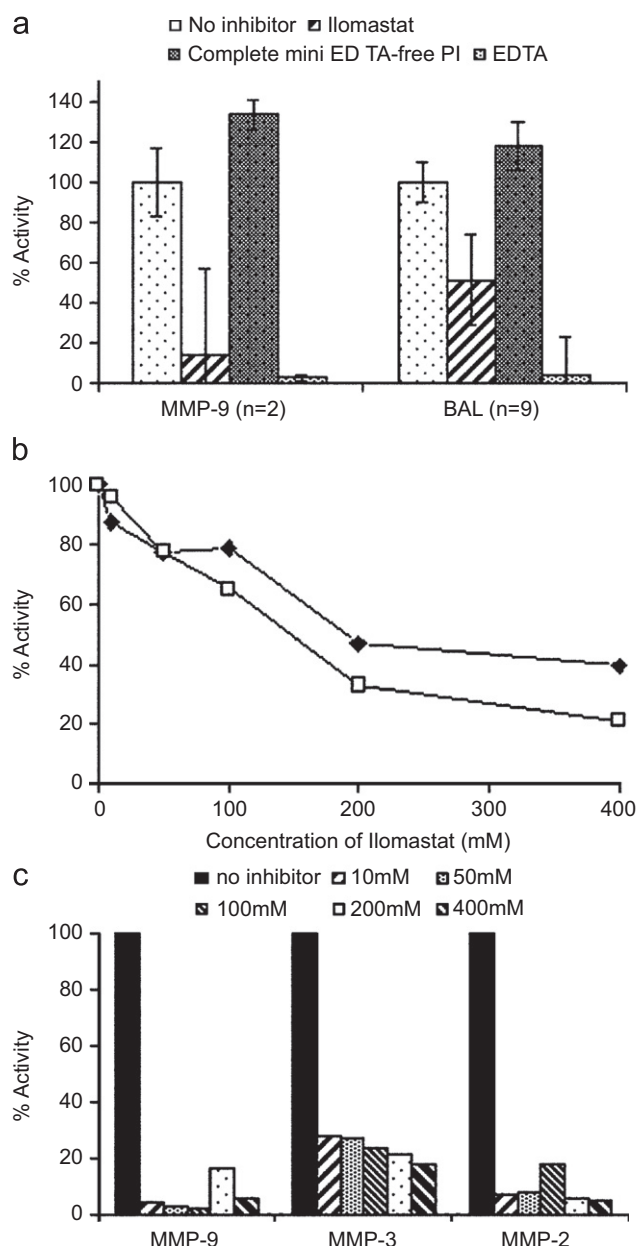


Figure 2 (a) The effect of protease inhibitors on MMP activity in BAL samples. Error bars denote standard deviations, $**p < 0.001$. (b) Effect of increasing concentrations of Ilomastat (a specific MMP inhibitor) on the activity measured in BAL from COPD subjects (◆) and smokers without COPD (□). (c) Effect of Ilomastat on the activity measured due to recombinant proteins MMP-9, MMP-3 and MMP-2.

Total MMP activity

Surprisingly total MMP activity, measured using the quench fluorescence assay, was equal in the COPD and HS groups (Figure 3a), and although not significant there was a trend towards lower MMP activity in those with the disease. Worsening airflow obstruction, measured by FEV_1/FVC ratio, was associated with lower MMP activity ($p = 0.016$, Figure 3b). Furthermore there appeared to be a trend towards lower MMP activity with increasing breathlessness as judged by MRC dyspnoea score (Figure 3c). To test for confounding by unequal sample concentration during the sputum induction we adjusted all the samples to the same protein concentration. The relationship between FEV_1/FVC ratio and total MMP activity persisted after protein correction ($p = 0.008$). In addition, mean total MMP activity was significantly lower in COPD patients after protein correction ($p = 0.03$). Lower total MMP activity was also associated with lower FEV_1 ($p = 0.05$) and higher MRC dyspnoea score ($p = 0.025$).

Collagenase, elastase and gelatinase activity

To further explore the finding of the quenched fluorescence assay, we used three independent MMP activity assays. These assays examined different MMP substrate specificities (collagenase, gelatinase and elastase) and therefore provide an alternative method of measuring MMP activity. Sputum collagenase, gelatinase and elastase activities were not associated with the presence of COPD, FEV_1 , or FEV_1/FVC ratio (Figure 4).

Assessment of TIMP-1 and TIMP-2 in induced sputum

A possible explanation for the reduced MMP activity despite increased MMP-9 protein as seen in those with COPD is an increase in MMP inhibitors. As previous data suggested TIMP-1 is raised in COPD subjects,^{10,13} we examined TIMP-1 and TIMP-2 mRNA and protein in our samples. RNA was extracted from induced sputum from 6 COPD subjects and 8 HS. RT-PCR for TIMP-1 and TIMP-2 were positive in all samples tested. Western blotting showed TIMP-1 protein was present in sputum samples, TIMP-2 protein was not detected (Figure 5). To quantitatively examine TIMP-1 expression we used an ELISA. No association was found between the levels of TIMP-1 and FEV_1 , FEV_1/FVC or MMP activity.

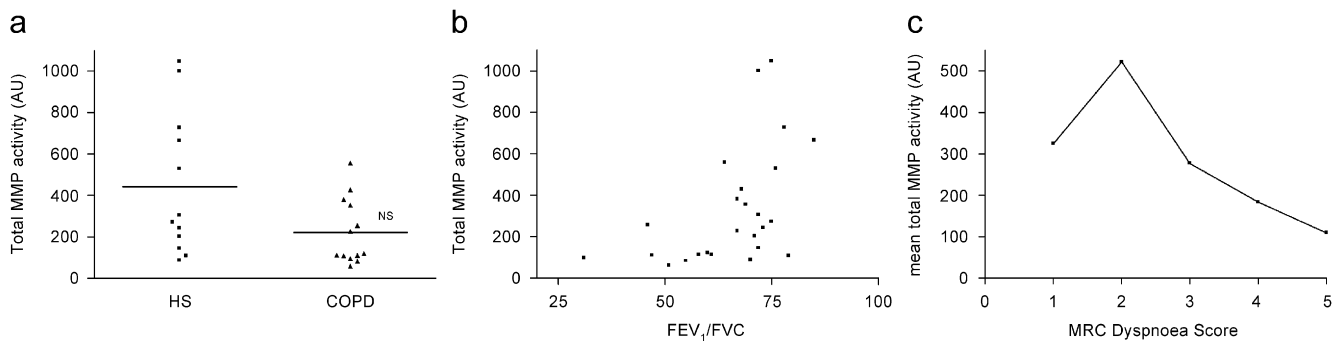


Figure 3 Total MMP activity measured using the quenched fluorescence assay in sputum related to the disease status, MRC dyspnoea score and FEV₁/FVC: (a) ANOVA comparing MMP activity in COPD and healthy smokers (HS) (NS = not significant). (b) Association of FEV₁/FVC and total MMP activity, $p = 0.016$. (c) Trend relationship between disease severity assessed using the MRC breathlessness score and total MMP activity.

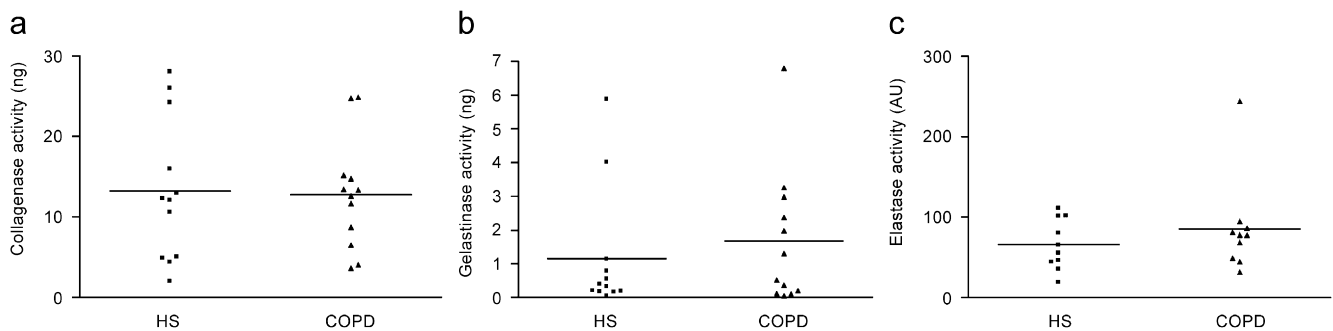


Figure 4 Collagenase, gelatinase and elastase activity in induced sputum in smokers without COPD (HS) and smokers with COPD (COPD), no significant differences were found between the two groups.

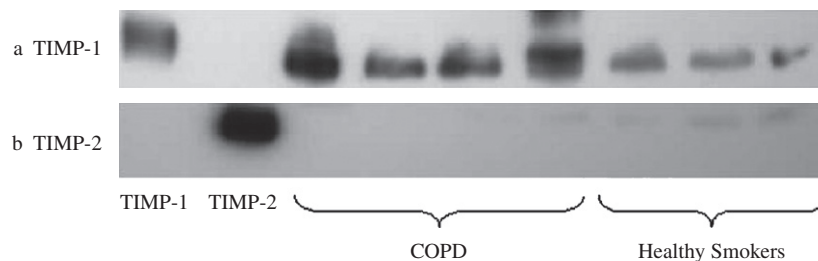


Figure 5 Western blot of induced sputum supernatant to identify (a) TIMP-1 and (b) TIMP-2 proteins. Blots were exposed for 15 min without any evidence of an appropriate size band that would be consistent with TIMP-2.

Discussion

Consistent with other studies we found higher levels of MMP-9 protein associated with COPD,^{13,14} but surprisingly found no significant difference in total MMP activity between smokers with COPD and smokers without COPD. Indeed there was a trend toward lower MMP activity in COPD and a significant negative correlation between total MMP activity and the degree of airflow obstruction.

Previous studies have shown increased levels of individual MMPs in patients with COPD.^{10,11,13,14,16,17} However, MMP protein levels do not directly reflect MMP activity as only the inactive form of the protein may be present, or an excess of an inhibitor may block activity. The experimental methods used in some studies may separate the protease from the inhibitor or may not take into account the amount of protein

in the active form. Our study adds to the previously published work by using a quenched fluorescence substrate that is only cleaved by activated MMPs and does not measure pro-MMPs or those complexed with an inhibitor,²⁴ thus providing a direct measure of the biological activity of metalloproteinases in the airways. Consistent with our observations, specific gelatinase, collagenase and elastase activities also showed no increase in MMP activity in COPD.

Experiments were performed to confirm that the proteolytic activity measured by the quench fluorescent assay was due to MMPs. The activity measured was unaffected by serine, cysteine and acidic protease inhibitors, but inhibited completely by the metal chelator EDTA suggesting that the activity is due to metalloproteinases. Activity was reduced with increasing concentrations of Ilomastat, a specific MMP inhibitor. The response to Ilomastat was dose dependent and

experiments using recombinant MMP protein showed MMP-3 to be less sensitive to ilomastat than MMP-2 or MMP-9, in keeping with the higher K_i of ilomastat for MMP-3 compared with those of MMP-2 and MMP-9. However, some activity remained even at high concentrations of ilomastat raising the possibility that other calcium- or zinc-dependent proteases, such as the ADAMs (a disintegrin and metalloproteinase), ADAMTSs (a disintegrin and metalloproteinase with a thrombospondin domain) or other uncharacterized metalloproteinase may be contributing to some of the activity measured. The activity of an uncharacterized metalloproteinase is in keeping with our observation that gelatinase, collagenase and elastase activities were not different between the two groups.

Our findings that total MMP activity measured using the quenched fluorescence assay was not raised in sputum of COPD patients compared with HS and that lower total MMP activity was associated with increasing airflow obstruction (FEV_1/FVC) ($p = 0.016$) are surprising in light of previous studies. Several important differences between our and other studies may be responsible. Firstly, the global MMP activity assay has not, to our knowledge, been used in sputum from COPD patients and may detect the metalloproteinase activity of enzymes not measured by specific ELISA or specific activity assay in other studies; for example due to reduced activity of a previously uncharacterized metalloproteinase, including an ADAM or ADAM-TS protein. Secondly, in contrast to some other studies, we studied smoking controls without COPD rather than non-smokers to eliminate the effect of smoking. Smoking, in addition to COPD, is likely to affect MMP activity as smoking results in increased airway neutrophils, a source of MMPs including MMP-9.

There are several potential mechanisms which could account for the discrepancy between protein expression and activity we observed; namely an increase in MMP inhibitors, reduced MMP activation, reduced activity of a previously uncharacterized metalloproteinase in COPD or activation of MMPs at a specific time or site (such as the cell surface) not sampled in our study.

The presence of increased MMP inhibitors in COPD is possible. Previous studies have demonstrated that TIMP-1 protein was inversely correlated with reduced FEV_1 in COPD.^{14,16} Although it has been suggested that the balance between protease-antiprotease is shifted toward increased proteolytic activity on the basis of elevated levels of MMP-9 in COPD,^{10,13} in studies that have analysed the MMP-9/TIMP-1 molar ratio it has been found that the balance is toward the antiprotease.^{14,16} Although other studies have shown higher levels of TIMP-1 in COPD compared to non-smoking controls our data did not show any significant relationship between TIMP-1 and smokers with and without COPD. In addition we were unable to detect significant TIMP-2 protein in our samples. The lower total MMP activity could still be explained by increased levels of other inhibitors not investigated as part of this study, for example TIMP-3 and TIMP-4.

Another potential explanation for our findings is reduced activation of MMPs in stable COPD. A study by Mercer et al. investigated MMP-9/TIMP-1 balance in stable disease and during exacerbations. They reported the MMP-9/TIMP-1 balance in favour of TIMP-1 in stable COPD, however, they found elevated levels of MMP-9 in the sputum of COPD

subjects during exacerbations, shifting the balance between MMP-9 and TIMP-1 in favour of MMP-9.²⁷ They suggested that there might be a shift between a "proteolytic" phenotype during an exacerbation and a "fibrotic" phenotype outside an exacerbation with the shift between "fibrotic" and "proteolytic" phenotypes contributing to the airway remodelling seen in COPD. Because the quenched fluorescence assay measures the dynamic balance between activated MMP and TIMP this could explain our findings and may reconcile it with other studies which have showed increased MMP and TIMP levels individually.

In summary, we have examined MMP activity by a variety of methods in induced sputum in patients with stable COPD and smokers without COPD. Despite increased MMP-9 protein, total MMP activity was not higher in sputum from COPD subjects. These findings may reflect raised levels of MMP inhibitors or a reduction in the activation of MMPs during stable disease, however, we have not been able to demonstrate this in the current study. Our findings suggest that the activity of MMPs in COPD may be more complex than previously thought and that the balance between MMPs and TIMPs, the course of disease including exacerbations, site of MMP activity and the overall effect on proteolytic activity needs to be considered when investigating airway inflammation.

Conflict of interest statement

The authors have no financial interest in the subject matter contained in the study. The authors declare that they have no competing interests.

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